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Spoilage potential of *Paenibacillus* sp. in Brazilian raw milk

Potencial deteriorante de *Paenibacillus* sp. no leite cru refrigerado brasileiro

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– NOTE –

ABSTRACT

Bacterial spores are widespread in the environment and can contaminate milk. Spores are resistant to thermal conditions and their germination reduces milk shelf-life because the aerobic bacteria that are sporulated produce proteases and lipases. The aim of this study was identify *Paenibacillus* sp., the spoilage microbiota, arising from the germination of spores in raw milk and your spoilage potential. Twenty different milk samples were treated at 80°C/12min and plated to isolate spore-forming bacteria. These strains were picked in milk agar and tributyrin agar for verification of their potential proteolytic and lipolytic activities, respectively. Amplification and sequencing of the 16S rRNA gene of the strains for identification by similarity to the DNA sequences deposited in GenBank was performed. One hundred and thirty-seven isolates were obtained, of which 40 (29.2%) showed spoilage activity for milk. Of these, three (7.5%) were identified as strains of *Paenibacillus* sp., and all were lipolytic. *Paenibacillus* sp. have been identified as primarily responsible for the spoilage of pasteurized milk with a long shelf-life in other countries. To increase the shelf-life of Brazilian pasteurized milk, it is important to identify the sporulated microbes to determine their origin and to control the contamination of milk by vegetative forms such as spores.

Key words: lipolysis, proteolysis, shelf-life, spores.

RESUMO

Os esporos de bactérias estão amplamente difundidos no ambiente e podem contaminar o leite. Os esporos conferem resistência aos processos térmicos e sua germinação pode reduzir a vida útil do leite pasteurizado, uma vez que bactérias esporuladas aeróbias são muito frequentemente produtoras de proteases e lipases. O objetivo do presente trabalho foi identificar *Paenibacillus* sp. na microbiota deteriorante, oriunda da germinação de esporos no leite cru, e avaliar o seu potencial

deteriorante. Vinte amostras de leite de propriedades distintas foram tratadas a 80°C/12min e semeadas para isolamento de bactérias formadoras de esporos. Essas cepas foram repicadas em ágar leite e tributirina para verificação do seu potencial proteolítico e lipolítico, respectivamente. Foi realizada a amplificação e sequenciamento do gene 16S do rRNA para identificação das cepas por similaridade com as sequências de DNA depositadas no GenBank. Foram obtidos 137 isolados, dos quais 40 (29,2%) apresentaram atividade deteriorante do leite. Dessas, 3 (7,5%) cepas foram identificadas como *Paenibacillus* sp., todas lipolíticas. *Paenibacillus* sp. são considerados os principais responsáveis pela deterioração do leite pasteurizado de longa vida útil em outros países. Para o aumento da vida útil do leite pasteurizado brasileiro, é importante conhecer a microbiota esporulada, de forma a determinar sua origem e poder controlar a contaminação do leite tanto pelas formas vegetativas como por seus esporos.

Palavras-chave: esporos, proteólise, lipólise, vida útil.

Bacterial spores are commonly found in soil (VISSERS et al., 2007) and in animal feed such as corn silage (BUEHNER et al., 2014). Spores can contaminate milk during and after milking, and then may germinate or remain in the spore form. Spores are resistant to heat treatment, which can stimulate germination. Sporulated bacteria that produce proteolytic and lipolytic enzymes are important in the spoilage process of long-life pasteurized milk (GLEESON et al., 2013) and ultra-high temperature milk (ESPEJO et al., 2014). Some of these

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microorganisms are psychrotrophic (HULL et al., 1992) or thermophilic (YUAN et al., 2012; BUEHNER et al., 2014).

In the United States, where pasteurized milk can have a shelf-life of 28 days for control of spoilage microorganisms in raw milk, *Paenibacillus* sp. spores yet are the largest contributors to the spoilage (HUCK et al., 2007). However, studies of Brazilian raw milk microbiota have not reported the isolation of this genera. Thus, the aim of this study was to verify the presence of micro-organisms of *Paenibacillus* sp. in refrigerated raw milk from the dairy region of Castro, Paraná, Brazil, and determine the spoilage potential.

Twenty samples of refrigerated raw milk were evaluated between November 2013 and May 2014. The samples were aseptically collected directly from the milk cooling tanks of different property. Treatment of the milk for the isolation of aerobic spores was performed according to the Standard Methods for the Examination of Dairy Products (FRANK et al., 2004).

Bacterial colonies from the spore germination were picked in milk agar (Acumedia, Baltimore, USA) supplemented in the ratio of 9:1 with sterile nonfat dry milk solution reconstituted to 10% w/v, and tributyrin agar (Himedia, Mumbai, India) supplemented in the ratio of 99:1 with tributyrin (Himedia, Mumbai, India). These media were used to verify proteolytic (BEERENS et al., 1990) and lipolytic activities (HANTSIS-ZACHAROV et al., 2007), respectively.

DNA was extracted from the colonies that showed spoilage activity following the method of CHENG et al. (2006). The 16S rRNA gene was amplified using the Y1f and Y3r primers (CHEN et al., 2000) in a thermocycler (Aeris™ Thermal Cycler, Esco® Micro Pte, Singapore). The amplification products were subjected to DNA sequencing by the Sanger method with the primers 27f and 1492r (OSBORNE et al., 2005) in an automatic sequencer (ABI 3500 Genetic Analyzer, Applied Biosystems, Carlsbad, CA, USA).

The chromatograms obtained in the sequencing were analyzed with the platform “Electropherogram quality analysis” <<http://asparagin.cenargen.embrapa.br/phph/>>. The quality of the sequences was analyzed using Phred. Then, the contig was obtained using the CAP3 program “Sequence Assembly Program” <<http://pbil.univ-lyon1.fr/cap3.php>> (HUANG et al., 1999). The contig was compared to other sequences

deposited in public database (GenBank) using the BLAST (Basic Local Alignment Search Tool - <<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>>) for similarity identification. The alignment of the nucleotide sequence with sequences from standard strains was estimated by CLUSTAL W (version 1.4) using the software package MEGA 6 (TAMURA et al., 2013). Phylogenetic analysis was calculated using the distance evolutionary model Tamura-Nei (TAMURA et al., 1993) and the Neighbor-joining algorithm with 1000 bootstrap replicates.

One hundred and thirty-seven pure isolates of spore-forming bacteria were obtained, of which 40 (29.2%) had spoilage activity in milk. Of these, three strains were identified as *Paenibacillus* sp., which corresponds to 7.5% of sporulated aerobic microbes with spoilage activity in the samples. All isolates of *Paenibacillus* sp. had lipase activity and none were proteolytic.

The 16S rRNA gene did not allow the identification of isolate similarity to the species level compared to other sequences deposited in GenBank. However, a phylogenetic analysis of the three sequences (select for deposit in the GenBank strain LIPOA/UEL_7 - Accession Number: KP713766) grouped together with other isolates of *Paenibacillus macerans* is shown in figure 1.

Microorganisms of the *Paenibacillus* genus are often reported as members of the spores and spoilage microbiota of raw milk in the United States (HUCK et al., 2007; RANIERI et al., 2012). This genus had not previously been identified in Brazilian milk microbiota; therefore, there are no reports of its spoilage activity in Brazilian milk. The origin of the contamination of milk by *Paenibacillus* sp. may be related to food animals, which are a source of contamination by *Bacillus* sp. (GIFFEL et al., 2002).

Germination of the spores of *Paenibacillus* sp. in pasteurized milk occurs during the shelf-life. Germination begins the process of spoilage, causing sensory changes (SCHELDEMAN et al., 2005) and promoting lipolysis and proteolysis. However, in this study, only lipolytic activity was observed. HUCK et al. (2007) also found that raw milk from the United States, experimentally pasteurized and stored for 14 days at 6°C, contained microbiota predominantly composed of *Paenibacillus* sp. (83 of 88 isolates).

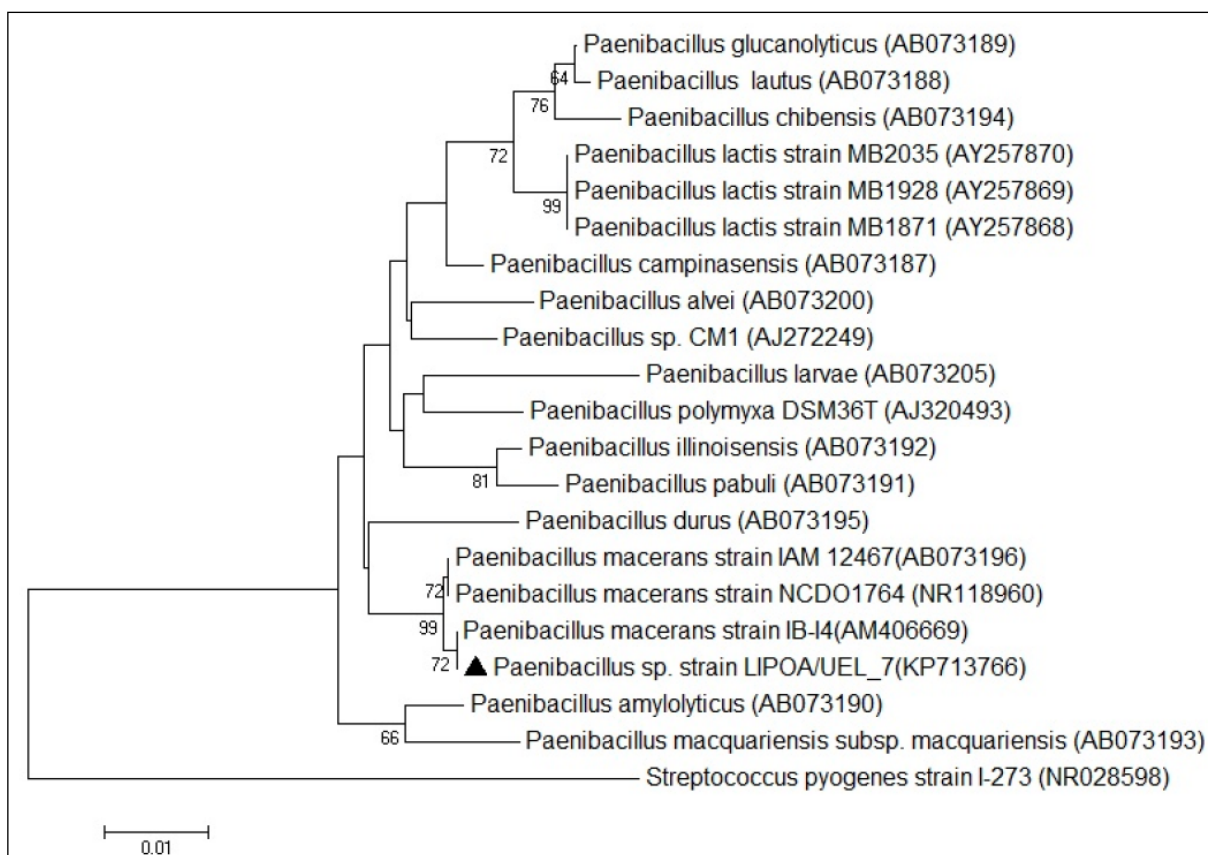


Figure 1 - Phylogenetic tree constructed using the neighbor-joining method based on the alignment of 665bp of the 16S rRNA gene of *Paenibacillus*. The bootstrap values are represented by the percentage of 1000 replications. The study samples (KP713766) is grouped in the same phylogenetic position as the strains of *Paenibacillus macerans* (AB073196, NR118960 and AM406669). The sequence of the 16S rRNA gene of *Streptococcus pyogenes* (strain I-273) was used as outgroup. The bar indicates 0.01 replacement of nucleotides.

Because spores are highly resistant to adverse environmental conditions and are easily spread through the air (TORTORA et al., 2013), it was expected that the Brazilian refrigerated raw milk would also contain *Paenibacillus* sp.

To increase the shelf-life of Brazilian pasteurized milk in some regions, such as this sample unit where the environmental contamination of raw milk is very low and there is the potential for the production of long shelf-life pasteurized milk, the control of spoilage microorganisms are of fundamental importance (GLEESON et al., 2013). The genetic identification of *Paenibacillus* sp. in Brazilian raw milk described in this study allows the realization of studies determining the source of the contamination and its real influence on the shelf-life of Brazilian pasteurized milk, ongoing studies in other countries (BUEHNER et al., 2014).

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